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KFERQ Sequence in Ribonuclease A-mediated Cytotoxicity*

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Onconase® (ONC) is an amphibian ribonuclease that is in clinical trials as a cancer chemotherapeutic agent. ONC is a homolog of ribonuclease A (RNase A). RNase A can be made toxic to cancer cells by replacing Gly^{88} with an arginine residue, thereby enabling the enzyme to evade the endogenous cytosolic ribonuclease inhibitor protein (RI). Unlike ONC, RNase A contains a KFERQ sequence (residues 7–11), which signals for lysosomal degradation. Here, substitution of Arg^{10} of the KFERQ sequence has no effect on either the cytotoxicity of G88R RNase A or its affinity for RI. In contrast, K7A/G88R RNase A is nearly 10-fold more cytotoxic than G88R RNase A and has more than 10-fold less affinity for RI. Up-regulation of the KFERQ-mediated lysosomal degradation pathway has no effect on the cytotoxicity of these ribonucleases. Thus, KFERQ-mediated degradation does not limit the cytotoxicity of RNase A variants. Moreover, only two amino acid substitutions (K7A and G88R) are shown to endow RNase A with cytotoxic activity that is nearly equal to that of ONC.

Onconase® (ONC)[1] is a homolog of ribonuclease A (RNase A; EC 3.1.27.5) from the Northern leopard frog, *Rana pipiens* (1, 2). ONC demonstrates both antitumor and antiviral activity, and is in Phase III clinical trials for the treatment of malignant mesothelioma (for reviews, see Refs. 3–8). The most damaging side effect from ONC treatment is renal toxicity, which is dosage-dependent and reversible (9, 10). Studies in mice have demonstrated that ONC is retained in the kidneys, in contrast to mammalian ribonucleases (11). Hence, mammalian ribonucleases could provide a ribonuclease-based anticancer therapy without renal toxicity.

ONC and RNase A share 30% amino acid sequence identity and have similar tertiary structure (2, 12), yet differ in catalytic activity and affinity for the cytosolic ribonuclease inhibitor protein (RI). RNase A demonstrates 10^4-fold more ribonucleolytic activity than does ONC (2, 13). In cells, the high ribonucleolytic activity of RNase A is blocked by its high affinity for RI (14–16) (Fig. 1). ONC has low affinity for RI (13, 17).

Several studies have focused on understanding the contribution of ribonucleolytic activity and affinity for RI to cytotoxicity. RNase A itself does not have marked antitumor activity, but variants of RNase A are toxic to cancer cells. For example, substituting the glycine residue at position 88 with arginine decreases the affinity for RI and endows RNase A with cytotoxic activity (16). Replacing Lys^{41} with an arginine residue results in a decrease in catalytic activity that is compensated by a decrease in affinity for RI (18). K41R/G88R RNase A has enhanced toxicity to K-562 cells as compared with G88R RNase A.

Some endosomal pathways end in the lysosomal degradation of proteins. The KFERQ pentapeptide sequence targets cytosolic proteins for lysosomal degradation via an alternative pathway (for reviews, see Refs. 19–21). Dice and co-workers (22) found that microinjected RNase A associates with lysosomes upon cellular fractionation. Subsequent studies found that the KFERQ pentapeptide, which comprises residues 7–11 of RNase A, regulates lysosomal degradation (23, 24). The KFERQ sequence of RNase A is recognized by a cytosolic heat shock cognate (hsc) protein of 73 kDa (hsc73) (25). ONC does not contain a KFERQ sequence (Fig. 2). The significance of KFERQ-targeted lysosomal decay in ribonuclease-mediated cytotoxicity is unknown. This sequence, along with RI, could serve to protect cells against an invading ribonuclease.

Here, we determine the effect of the KFERQ sequence on G88R RNase A-mediated cytotoxicity. Replacing Lys^7 of the KFERQ sequence with an alanine residue has little effect on the conformational stability or catalytic activity of G88R RNase A. K7A/G88R RNase A does, however, have a marked decrease in affinity for RI compared with G88R RNase A and is the most cytotoxic variant of RNase A reported to date. Using other RNase A variants with substitutions in the KFERQ sequence that do not disrupt RI binding, we find that targeted lysosomal degradation via the KFERQ sequence does not modulate ribonuclease toxicity. Moreover, the toxicity of ribonucleases is not diminished in serum-deprived cells, which have enhanced KFERQ-mediated lysosomal degradation (22).

EXPERIMENTAL PROCEDURES

Materials—K-562 cells, which derive from a continuous human chronic myelogenous leukemia line, were from the American Type Culture Collection (Manassas, VA). Cell culture medium and supplements were from Invitrogen.

ONC (16) and porcine RI (26) were prepared as described. Enzymes used for DNA manipulation were from Promega (Madison, WI) or New England Biolabs (Beverly, MA).

[*methyl*-^{3}H]Thymidine was from PerkinElmer Life Sciences (Boston, MA). 6-Carboxyfluorescein~dArU$(dA)_2$~6-carboxytetramethylrhodamine (6-FAM~dArU$(dA)_2$~6-TAMRA) was from Integrated DNA Technologies (Coralville, IA). Yeast rRNA (16 S and 23 S) was from Roche Molecular Biochemicals. All other chemicals were of commercial reagent grade or better and were used without further purification.

Analytical Instruments—Ultraviolet and visible absorption was measured with a Cary model 50 spectrophotometer from Varian (Sugar Land, TX). Fluorescence was measured with a QuantaMaster1 photon-counting spectrofluorometer from Photon Technology International

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[1] The abbreviations used are: ONC, Onconase® (a registered trademark of Alfacell, Inc.); 6-FAM, 6-carboxyfluorescein; RI, ribonuclease inhibitor; 6-TAMRA, 6-carboxytetramethylrhodamine; PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid.

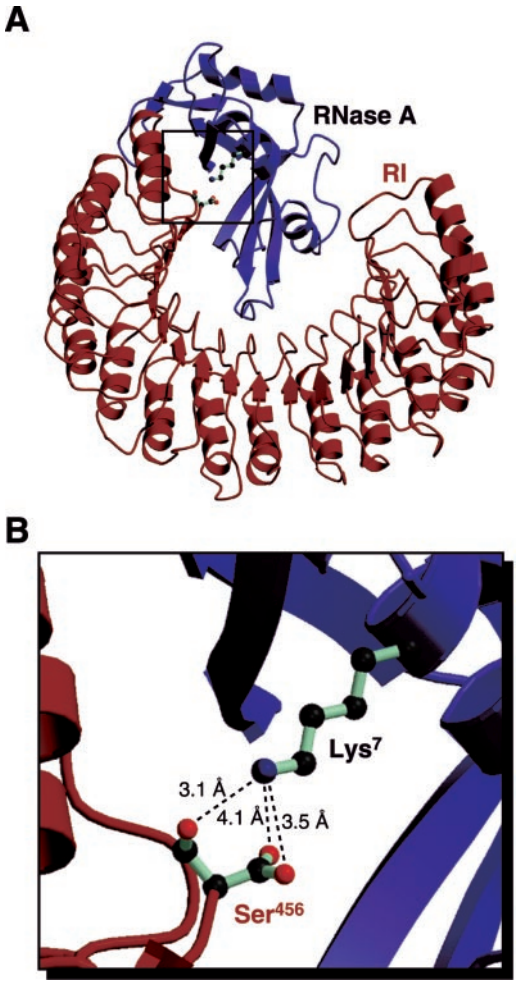

FIG. 1. **Molecular interactions between porcine ribonuclease inhibitor (*red*) and ribonuclease A (*blue*).** This figure was created by using atomic coordinates derived by x-ray diffraction analysis (39) and the program MOLSCRIPT (34). *A*, crystalline structure of the RI·RNase A complex. *B*, close-up of the interaction between Lys^7 (RNase A) and Ser^{456} (RI).

	7	10
RNase A	K E T A A A **K F E R Q** H M D S S T S A A	
Onconase®	– Q D W L T – F Q K K H I T N T R D V D	

FIG. 2. **Amino acid sequence of residues 1–20 of ribonuclease A and the corresponding residues of Onconase®.**

(South Brunswick, NJ), using fluorescence-grade quartz or glass cuvettes (1.0-cm path length, 3.0-ml volume) from Starna Cells (Atascadero, CA). Radioactivity was measured with a Beckman model LS 3801 liquid scintillation counter from Beckman Instruments (Fullerton, CA).

Production of Ribonuclease A Variants—Plasmid pBXR (27) directs the expression of wild-type RNase A in *Escherichia coli*. K7A RNase A and R10A RNase A were created by using oligonucleotide-mediated site-directed mutagenesis (28). The K7A/G88R and R10A/G88R substitutions within RNase A were created by ligating DNA fragments using the *Apa*I and *Mun*I restriction sites. The K7A/K41R/G88R RNase A variant was created by ligating the DNA fragments using the *Apa*I and *Cla*I restriction sites.

Protein Purification—Wild-type RNase A and its variants were purified by using methods described previously (16, 27, 29, 30), but with the following modifications. Refolding solutions for RNase A variants with the G88R substitution contained 0.50 M arginine, instead of 0.10 M NaCl. Protein concentrations were determined by UV spectroscopy using $\epsilon = 0.72$ ml mg^{-1} cm^{-1} at 277.5 nm for RNase A (31) and its variants and $\epsilon = 0.87$ ml mg^{-1} cm^{-1} at 280 nm for ONC (16). All ribonucleases used in biological assays were dialyzed exhaustively *versus* phosphate-buffered saline (PBS), which contained in 1.00 liter: KCl (0.20 g), KH_2PO_4 (0.20 g), NaCl (8.0 g), and Na_2HPO_4 (2.16 g).

Assay of Conformational Stability—The conformational stability of RNase A variants was determined by monitoring the absorbance at 287 nm (A_{287}) with increasing temperature (32). The temperature of solution of protein (0.3 mg/ml) in PBS was increased from 25 to 75 °C in 1 °C increments. The A_{287} was recorded after a 7-min equilibration at each temperature. The value of T_m is the temperature at the midpoint of the thermal denaturation. Data were collected and analyzed with the program THERMAL (Varian Analytical Instruments; Walnut Creek, CA).

Assay of Catalytic Activity—Ribonucleolytic activity was measured by using a fluorogenic substrate (33). Assays were performed at 23 °C in 2.00 ml of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M). Solutions contained 6-FAM~$dArU(dA)_2$~6-TAMRA (50 nM) and enzyme (1.0–5.0 pM). Fluorescence was monitored by using 493 and 515 nm for the excitation and emission wavelengths, respectively. Kinetic parameters were determined by a linear least-squares regression analysis of the initial velocity using Equation 1 (33),

$$V/K = \frac{(\Delta F/\Delta t)}{F_{max} - F_0} \qquad \text{(Eq. 1)}$$

where V/K is the first-order rate constant, $\Delta F/\Delta t$ is the slope from the linear regression, F_{max} is the final fluorescence intensity after the reaction has reached completion, and F_0 is the initial fluorescence intensity before enzyme is added. The value of k_{cat}/K_m was calculated by dividing V/K by the enzyme concentration.

Fluorescence Assay of Ribonuclease Inhibitor Binding—The value of K_d for the complex between porcine RI and RNase A variants was determined by using a competitive binding assay. It has been shown that the fluorescence of fluorescein-labeled A19C/G88R RNase A (fluorescein~G88R RNase A) decreases by ~15% upon binding to RI.[2] Thus, fluorescence spectroscopy can be used to evaluate the ability of an unlabeled ribonuclease to compete with fluorescein~G88R RNase A for binding to RI. Specifically, cuvettes of PBS containing fluorescein~G88R RNase A (50 nM), an unlabeled RNase A variant (1 nM–2 μM), and dithiothreitol (1 mM) were incubated at room temperature (23 ± 2 °C). After 15 min, the initial fluorescence intensity was measured with 490 and 511 nm for the excitation and emission wavelengths, respectively. Next, RI was added with stirring (to 50 nM), and the average fluorescence intensity was measured after an additional incubation of 4 min. The maximum fluorescence decrease upon RI binding was measured with samples that lacked unlabeled ribonuclease. The concentration of the RI·fluorescein~G88R RNase A complex was determined by comparing the fluorescence of a sample with the fluorescence decrease observed when all of the fluorescein~G88R RNase A was bound by RI. The K_d value was determined as described.[2]

Gel Assay of Ribonuclease Inhibitor Binding—The effect of RI binding on catalytic activity was monitored directly, but qualitatively, by an agarose gel-based assay as described previously (16). Briefly, 0.6-ml siliconized microcentrifuge tubes of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M), dithiothreitol (1 mM), yeast rRNA (4 μg), and a ribonuclease (10 ng) were mixed with RI (0, 10, 20, or 40 units, where 1 unit is the amount required to inhibit the activity of 5 ng of RNase A by 50%). After a 15-min incubation at 37 °C, 10 mM Tris-HCl buffer (pH 7.5) containing EDTA (50 mM), glycerol (30%, w/v), xylene cyanol FF (0.25%, w/v), and bromphenol blue (0.25%, w/v) was added. Samples were analyzed by electrophoresis through an agarose gel (1%, w/v) containing ethidium bromide (0.4 μg/ml). Control samples were incubated in the absence of a ribonuclease or RI (or both).

Assay of Cytotoxicity—The effect of RNase A, its variants, and ONC on cell proliferation was determined by measuring the incorporation of [*methyl*-^{3}H]thymidine into cellular DNA. K-562 cells were grown in RPMI 1640 medium. Unless indicated otherwise, all culture medium contained fetal bovine serum (10%, v/v), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were cultured at 37 °C in a humidified incubator containing CO_2 (g; 5%, v/v). All toxicity studies were performed using asynchronous log-phase cultures. For toxicity assays, cells (95 μl of a solution of 5×10^4 cells/μl) were incubated with a 5-μl solution of a ribonuclease or PBS in the wells of a 96-well plate. Cells were incubated for 44 h at 37 °C in a humidified incubator containing CO_2 (g; 5%, v/v). Next, the proliferation of cells was monitored with a 4-h pulse of [*methyl*-^{3}H]thymidine (0.4 μCi/well). Cells were harvested onto glass fiber filters using a PHD Cell Harvester (Cambridge Technology; Watertown, MA). Filters were washed with water and dried with methanol, and their ^{3}H content was measured with liquid scintillation counting.

[2] R. A. Abel, M. C. Haigis, C. Park, and R. T. Raines (2002), submitted manuscript.

TABLE I
Attributes of ribonuclease A, its variants, and Onconase®

Ribonuclease	T_m[a]	IC_{50}[b]	k_{cat}/K_m[c]	K_d[d]	$\Delta\Delta G$[e]
	°C	μM	(10^6 M^{-1} s^{-1})	nM	kcal/mol
RNase A	63	>25	43 ± 0.3	67×10^{-6}	0.0
G88R RNase A	63	7.3 ± 0.4	15 ± 3	0.54 ± 0.07	5.3
K7A RNase A	63	>25	9.2 ± 1.6	0.07 ± 0.01	4.1
K7A/G88R RNase A	62	1.0 ± 0.1	8.8 ± 2.6	7.2 ± 0.4	6.8
R10A RNase A	60	>25	8.2 ± 0.8	ND[f]	ND
R10A/G88R RNase A	62	6.9 ± 0.8	12 ± 1	0.56 ± 0.07	5.3
K41R/G88R RNase A	63	3.9 ± 0.4	0.41 ± 0.05	2.9 ± 0.4	6.3
K7A/K41R/G88R RNase A	63	12.3 ± 1.9	0.07 ± 0.01	47 ± 4	7.9
ONC	90	0.54 ± 0.04	0.00035 ± 0.00001	≥10^3	≥9.7

[a] Values of T_m (± 1 °C) were determined in PBS by UV spectroscopy. The T_m value of ONC is from Ref. 44 and was determined by CD spectroscopy.
[b] Values of IC_{50} are for incorporation of [methyl-^{3}H]thymidine into the DNA of K-562 cells.
[c] Values of k_{cat}/K_m (± S.E.) are for the catalysis of 6-FAM~dArU$(dA)_2$~6-TAMRA cleavage at 25 °C in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M).
[d] Values of K_d were determined at (23 ± 2) °C in PBS. The K_d value for RNase A is from Ref. 15. The K_d value for ONC is an estimate from Ref. 13.
[e] Values of $\Delta\Delta G$ were calculated with the equation: $\Delta\Delta G = -RT\ln(K_d^{\text{RNase A}}/K_d)$.
[f] ND, not determined.

Serum-deprived cells have enhanced KFERQ-mediated lysosomal degradation of RNase A (22). To discern the effect of RNase A, its variants, and ONC on the proliferation of cells with enhanced KFERQ-mediated degradation, K-562 cells were grown in RPMI medium without fetal bovine serum for 18 h prior to the addition of a ribonuclease. Ribonuclease-mediated cytotoxicity was measured as described above.

Cytotoxicity data were analyzed with the programs SIGMAPLOT (SPSS Science; Chicago, IL) and DELTAGRAPH (DeltaPoint; Monterey, CA). Each data point represents the mean (± S.E.) of at least three experiments, each performed in triplicate. The IC_{50} value of each variant was calculated by using Equation 2,

$$S = \left(\frac{IC_{50}}{IC_{50} + [\text{ribonuclease}]}\right) \times 100 \qquad \text{(Eq. 2)}$$

where S is the percent of total DNA synthesis after the incubation period (48 h).

RESULTS

Design of Ribonuclease A Variants—RNase A variants were designed with the primary goal of discerning a role for the KFERQ sequence (residues 7–11) in cytotoxicity. Because RNase A itself is not cytotoxic, the cytotoxic G88R RNase A variant was used as a basis for this work. In addition, amino acid substitutions were combined with the secondary goal of producing variants that have high ribonucleolytic activity, low affinity for RI, and thus (presumably) high cytotoxicity.

Lys^7 and Arg^{10}—Lys^7 and Arg^{10} comprise the enzymic P2 subsite, which interacts with a phosphoryl group of an RNA substrate (35–37). Cuchillo and co-workers (35) found that replacing either Lys^7 or Arg^{10} alone with a glutamine residue has only a minor effect on catalysis of RNA cleavage, but that replacing both Lys^7 and Arg^{10} decreases catalytic activity by 60-fold. Likewise, replacing both Lys^7 and Arg^{10} with alanine residues results in a k_{cat}/K_m value that is 60-fold lower than that of wild-type RNase A (37).

The interaction between Lys^7 of RNase A and porcine RI was investigated previously by using semisynthetic variants (38). Neumann and Hofsteenge (38) found that replacing Lys^7 with an *S*-methyl cysteine residue resulted in a >50-fold decrease in affinity for RI. This result is consistent with the structure of the RI·RNase A complex in which the side-chain nitrogen of Lys^7 donates a hydrogen bond to the C-terminal carboxyl group of RI (Fig. 1) (39). Arg^{10} makes no contact with RI in the complex.

Accordingly, we replaced Lys^7 and Arg^{10} of RNase A independently with an alanine residue. The resulting K7A and R10A variants are designed to disrupt the KFERQ sequence, without decreasing ribonucleolytic activity. By systematically incorporating these changes in a cytotoxic variant, G88R RNase A, we were able to investigate the contribution of Lys^7 and Arg^{10} to cytotoxicity.

Phe^8, Glu^9, and Gln^{11}—Phe^8, Glu^9, and Gln^{11} are important to the structure and function of RNase A. Replacing Gln^{11} with an alanine, glutamine, or histidine residue enables the enzyme to bind a substrate in a nonproductive manner (40). The contributions of Phe^8 and Glu^9 have been determined in RNase S (or RNase S′), which is a noncovalent complex of residues 1–20 (or 1–15) and 21–124. In this complex, replacing Phe^8 (41) or Glu^9 (42) with other residues decreases its conformational stability or catalytic activity (or both). Because these three residues of the KFERQ sequence play roles other than in lysosomal degradation, we left them intact.

Lys^{41}—The side chain of Lys^{41} of RNase A donates a hydrogen bond to the transition state during catalysis of RNA cleavage (29). Changing Lys^{41} to an arginine residue results in a 10^2-fold decrease in catalytic activity. Although K41R/G88R RNase A has low catalytic activity, it binds RI with less affinity than does G88R RNase A. Moreover, K41R/G88R RNase A is more cytotoxic than G88R RNase A (18). Hence, we used the K7A/K41R/G88R RNase A variant to explore the additivity of single substitutions that disrupt RI binding, as well as the relationship between catalytic activity, RI affinity, and cytotoxicity.

Conformational Stability—The conformational stability of the RNase A variants was measured to ensure that the proteins were folded properly during all assays. Values of T_m for RNase A, its variants, and ONC are listed in Table I. The T_m values of both wild-type RNase A and G88R RNase A in PBS were determined to be 63 °C, respectively; this value is similar to those reported previously (16, 43). K7A RNase A, K7A/G88R RNase A, R10A RNase A, and R10A/G88R RNase A were found to have T_m values of 63, 62, 60, and 62 °C, respectively. K41R/G88R RNase A and K7A/K41R/G88R RNase A were both found to have a T_m value of 63 °C. Hence, all RNase A variants were essentially completely folded during assays at 37 °C or room temperature. The T_m value of ONC in PBS was reported previously to be 90 °C (44).

Catalytic Activity—The cytotoxicity of ribonucleases relies on their ribonucleolytic activity (17, 45, 18). Ribonucleolytic activity was measured by using a fluorogenic substrate, 6-FAM~dArU$(dA)_2$~6-TAMRA, which exhibits a nearly 200-fold increase in fluorescence upon cleavage of the P–$O^{5'}$ bond on the 3′ side of the single ribonucleotide-embedded residue

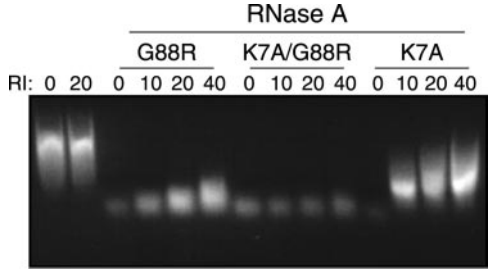

FIG. 3. **Agarose gel-based assay for inhibition of ribonucleolytic activity by porcine ribonuclease inhibitor**. Inhibition was assessed by visualizing the cleavage of rRNA by 10 ng of a ribonuclease in the absence or presence of RI (10, 20, or 40 units).

(33). Values of k_{cat}/K_m for RNase A, its variants, and ONC are listed in Table I. The values of k_{cat}/K_m for RNase A and ONC were 4.3×10^7 and 3.5×10^2 M^{-1} s^{-1}, respectively, which is in good agreement with those reported previously (33, 44). The values of k_{cat}/K_m for K7A RNase A and R10A RNase A were 9.2×10^6 and 8.2×10^6 M^{-1} s^{-1}, respectively, which are lower by 4–5-fold than those of the wild-type enzyme. These values are not reduced by the G88R substitution; the k_{cat}/K_m values for K7A/G88R RNase A and R10A/G88R RNase A were 8.8×10^6 and 1.2×10^7 M^{-1} s^{-1}, respectively. As expected, the K41R variants have greatly diminished catalytic activity. The k_{cat}/K_m value of 4.1×10^5 M^{-1} s^{-1} for K41R/G88R RNase A is in good agreement with the activity reported previously (18). The value of k_{cat}/K_m for K7A/K41R/G88R RNase A is 0.70×10^5 M^{-1} s^{-1}.

Ribonuclease Inhibitor Binding—RI is a modulator of intracellular ribonucleolytic activity. The values of K_d for the RI·RNase A, RI·G88R RNase A, and RI·fluorescein~G88R RNase A complexes were found previously to be 67 fM (15), 0.57 nM,[2] and 0.55 nM,[2] respectively. Here, the K_d values for the complex between RI and other RNase A variants were measured by using a competition assay (Table I). The K7A substitution disturbs the interaction of RI and RNase A, giving K_d values of 0.07 and 7.2 nM for K7A RNase A and K7A/G88R RNase A, respectively. In contrast, the R10A substitution has no significant effect on the affinity for RI, as the value of K_d for the RI·R10A/G88R RNase A complex is indistinguishable from that of the RI·G88R RNase A complex. The K_d value for the RI·K41R/G88R RNase A complex is 2.9 nM, which is comparable with the value of K_i = 3.0 nM reported previously (18). Addition of the K41R substitution to K7A/G88R RNase A decreases the affinity for RI by 5-fold, giving a K_d value of 47 nM for the triple variant. These K_d values were used to calculate the change in the free energy of interaction for RI and the RNase A variants. These values of $\Delta\Delta G$ are listed in Table I.

An agarose gel-based RI-evasion assay was used to verify that affinity for RI correlates with inhibition of catalytic activity. In this assay, ribonucleases were incubated with increasing amounts of RI in the presence of yeast rRNA as substrate. The samples were subjected to electrophoresis through an agarose gel, and degraded RNA was observed by its faster mobility and less efficient staining compared with control samples without ribonuclease. We find that K7A/G88R RNase A degrades yeast rRNA more thoroughly in the presence of RI than does either G88R RNase A or K7A RNase A (Fig. 3).

Cytotoxicity Assays—The toxicity of each ribonuclease was measured with the K-562 human leukemia cell line. The resulting IC_{50} values are listed in Table I. ONC, G88R RNase A, and K41R/G88R RNase A had IC_{50} values similar to those reported previously (16, 18). Like wild-type RNase A, K7A RNase A and R10A RNase A were not cytotoxic, even at protein concentrations of 25 μM. The cytotoxicity of R10A/G88R RNase A did not differ from that of G88R RNase A. In contrast, the

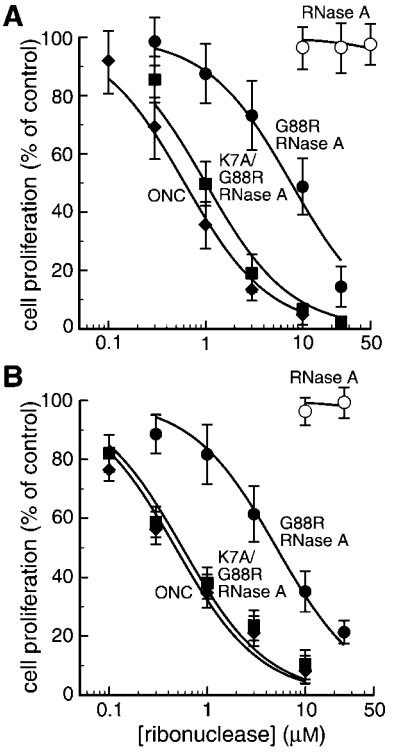

FIG. 4. **Effect of ribonucleases on the proliferation of K-562 cells.** Cell proliferation was measured by incorporation of [*methyl*-^{3}H]thymidine into cellular DNA after a 48-h incubation at 37 °C with a ribonuclease. Each value is the mean (± S.E.) of at least three independent experiments with triplicate samples and is expressed as a percentage of the PBS control. *A*, assays in the presence of fetal bovine serum (10%, v/v). *B*, assays on cells deprived of fetal bovine serum for 18 h prior to the addition of a ribonuclease.

IC_{50} value of K7A/G88R RNase A was 7-fold lower than that of G88R RNase A (Fig. 4*A*). The IC_{50} value of K7A/G88R RNase A was within 2-fold of that of ONC. Unexpectedly, the triple variant, K7A/K41R/G88R RNase A had an IC_{50} value that was similar to that of G88R RNase A, and 2- and 12-fold greater than that of K41R/G88R RNase A and K7A/G88R RNase A, respectively.

To test further whether KFERQ-mediated degradation contributes to differences between the cytotoxicity of ONC and RNase A variants, assays were performed with serum-deprived K-562 cells. The IC_{50} values of ONC, K7A/G88R RNase A, and G88R RNase A did not change significantly in serum-deprived cells as compared with control cells cultured with fetal bovine serum (Fig. 4*B*; Table II).

DISCUSSION

Secretory ribonucleases can be potent cytotoxins by virtue of their ability to enter the cytosol and catalyze the cleavage of cellular RNA (3–8). The ribonucleolytic activity within the cytosol is regulated by two factors: (i) the concentration of enzyme within the cytosol, and (ii) how much of that enzyme is bound by RI. The cytosolic concentration of a ribonuclease is dependent on the balance between its import and degradation. The ability of a ribonuclease to reach the cytosol is known to limit its toxicity. For example, even wild-type RNase A is toxic to cells when injected directly into the cytosol (46). ONC and variants of RNase A are more toxic to cells in the presence of drugs that alter intracellular trafficking (47).[3] The contribution of protein degradation to ribonuclease cytotoxicity has been less studied, but its importance can be inferred. For example,

[3] M. C. Haigis and R. T. Raines, unpublished results.

TABLE II
Toxicity of ribonuclease A, its variants, and Onconase® for serum-deprived cells

Ribonuclease	IC_{50}[a]
	μM
RNase A	>25
G88R RNase A	5.0 ± 0.4
K7A/G88R RNase A	0.62 ± 0.04
ONC	0.48 ± 0.03

[a] Values of IC_{50} are for incorporation of [*methyl*-^{3}H]thymidine into the DNA of K-562 cells grown in the absence of fetal bovine serum for 18 h prior to the addition of a ribonuclease.

ONC is more cytotoxic in the presence of protease inhibitors (48). In addition, the degradation of fluorescein-labeled RNase A in murine L cells can be blocked by preincubation with lysosomal protease inhibitors (30).

The sequence-specific, lysosome-targeted degradation of cytosolic proteins can also lower the cellular concentration of a protein. RNase A, unlike ONC, contains a KFERQ sequence (Fig. 2). This sequence is required for the targeted lysosomal degradation of cytosolic RNase A (22, 23). The targeted degradation of RNase A or toxic RNase A variants would lead to a decrease in cytosolic ribonucleolytic activity, and hence a decrease in cytotoxicity. Dice *et al.* (23) found that certain fragments of the RNase A KFERQ sequence (residues 1–10 and 2–8) are not degraded in a serum-dependent manner. In addition, although the order of the KFER residues is unimportant (20), the biochemical nature of the side chains must be conserved. Thus, replacing residues of the KFERQ sequence could enhance the cytotoxicity of an RNase A variant.

Arg^{10} of RNase A is located in the KFERQ sequence, but does not form any interaction with RI. Hence, we used the R10A variant to isolate the consequence of lysosomal degradation from RI evasion. R10A RNase A is not toxic to cells. In addition, R10A/G88R RNase A has an IC_{50} value similar to that of G88R RNase A. We also investigated the toxicity of a G88R RNase A variant with Gln^{11} replaced by a histidine or alanine residue. The cytotoxicity of the Q11H/G88R and Q11A/G88R variants does not differ from that of G88R RNase A (data not shown). Hence, disrupting the KFERQ sequence has no effect on ribonuclease-mediated cytotoxicity.

We measured the cytotoxicity of ribonucleases in cells with up-regulated lysosomal degradation. This experiment was based on the hypothesis that if KFERQ-mediated degradation limits the concentration of cytosolic ribonuclease, then enhancing this pathway would result in decreased toxicity. Cells cultured in the absence of serum show enhanced degradation of cytosolic RNase A (22). The data demonstrate that toxic variants of RNase A do not have lowered potency in serum-deprived cells (Fig. 4*B* and Table II). These results indicate that the KFERQ-mediated degradation of cytosolic ribonucleases does not limit their potency.

RI binds to members of the RNase A superfamily in a 1:1 stoichiometry (49). The interaction between RNase A and porcine RI buries 2550 Å^2 of protein surface and forms one of the tightest noncovalent complexes known, with K_d values in the fM range (14, 39, 50). Upon binding to RI, the activity of RNase A is abolished completely.

We have shown that Lys^7 of RNase A is an important residue in the RI-RNase A interaction. In the crystalline structure of the RI·RNase A complex, Lys^7 is proximal to Ser^{456} of porcine RI (Fig. 1*B*) (39), which corresponds to Ser^{460} of human RI. The distance between the side chain nitrogen of Lys^7 and the side-chain oxygen of Ser^{456} is 3.1 Å. The side-chain nitrogen is 3.5 and 4.1 Å away from the two oxygens of the C-terminal carboxyl group of RI. Replacing Lys^7 with an alanine residue removes any hydrogen bonds and favorable Coulombic interactions with Ser^{456} of RI. The value of K_d for the RI·K7A RNase A complex is 70 pM (Table I). The cooresponding value of K_d for the double variant, K7A/G88R RNase A, is 7.2 nM. Moreover, K7A/G88R RNase A is endowed with enhanced cytotoxicity.

Surprisingly, we find that the interactions of Lys^7, Lys^{41}, and Gly^{88} with RI are not additive. Single substitutions at Lys^7 or Gly^{88} result in decreases of binding free energy of 4.1 or 5.3 kcal/mol, respectively (Table I). The double variants, K7A/G88R RNase A and K41R/G88R RNase A, have lost 6.9 and 6.3 kcal/mol of binding free energy, respectively. Yet, the triple variant, K7A/K41R/G88R RNase A, has lost only 8.0 kcal/mol of binding free energy. If the interactions had been additive, then the effect of single substitutions would contribute fully to the loss of binding free energy. Such conservation in binding free energy loss would suggest rigidity between the interface of the complex. For example, the effect of single substitutions at Lys^1 and Lys^7 of RNase A on RI binding is indeed additive, suggesting that changing Lys^1 does not affect RI binding interactions at Lys^7 (38). In contrast, our data show that the interface between RI and RNase A is not rigid. Rather, compensatory changes occur upon perturbation of key contacts. A similar conclusion was reached by Shapiro *et al.* (51) who measured the affinity of RI variants for wild-type RNase A. Thus, the dynamic nature of the RI-RNase A interface must be addressed when engineering new ribonuclease variants to evade RI.

In conclusion, we have shown that the KFERQ sequence does not contribute to a decrease in ribonuclease-mediated cytotoxicity. We find that Lys^7 of RNase A contributes to a key interaction that tethers the N terminus of RNase A with the C terminus of RI. The K7A/G88R RNase A variant has $>10^5$-fold lower affinity for RI than does wild-type RNase A. The ribonucleolytic activity of K7A/G88R RNase A is, however, within 10-fold of that of the wild-type enzyme. Together, its high ribonucleolytic activity and low affinity for RI make K7A/G88R RNase A the most cytotoxic known variant of RNase A, with an IC_{50} value within 2-fold of that of ONC. Finally, we have found that the RI-RNase A interface is dynamic; disruption of one contact can alter other contacts.

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